TITLE OF THE INVENTION

PLASMID AUTONOMOUSLY REPLICABLE IN CORYNEFORM BACTERIA

BACKGROUND OF THE INVENTION

The present invention relates to a novel plasmid derived from *Corynebacterium thermoaminogenes*. The plasmid of the present invention can be utilized for improving ~~of~~ coryneform bacteria, which are used ~~as bacteria~~ for producing useful substances such as L-amino acids.

Amino acids, including L-glutamic acid and L-lysine, are produced by fermentative methods using the ~~so-called~~ coryneform bacteria, which generally belong to the genus *Brevibacterium*, *Corynebacterium* or *Microbacterium*, or variant strains thereof (Amino Acid Fermentation, Gakkai Shuppan Center, pp.195-215, 1986).

In the industrial fermentative production of amino acids, besides improve~~menting~~ing ~~in~~ the yield relative to saccharides, shortening ~~of~~ the culture time, improve~~menting~~ing ~~in~~ the amino acid ~~accumulation~~ concentration, and so forth, ~~use of an elevated~~ increasing the culture temperature is ~~considered~~ an important ~~as a~~ technical factor that ~~raises~~ increases the economical efficiency. That is, the culture is usually performed at an optimum fermentation temperature, ~~and the optimum temperature~~ which is 31.5°C for *Corynebacterium glutamicum*. After the culture is started, heat is generated during the fermentation, and hence amino acid production is markedly reduced if this heat output is not removed. Therefore, cooling

equipment is required in order to maintain the optimum temperature of the culture broth ~~to be optimum~~. On the other hand, if the culture temperature can be elevated, it ~~becomes~~ is then possible to decrease the energy required for cooling and the cooling equipment can be ~~made small~~ reduced in size.

Among coryneform bacteria, *Corynebacterium thermoaminogenes* has been isolated as a coryneform bacterium that can grow in ~~a~~ higher temperatures ~~region~~ (Japanese Patent Application Laid-open (Kokai) No. 63-240779). Whereas growth of *Corynebacterium glutamicum* is markedly suppressed at 40°C, *Corynebacterium thermoaminogenes* can grow at a temperature of about 40°C or higher, and is ~~considered~~ therefore ~~to be~~ suitable for high temperature fermentation.

Currently, ~~improving relying~~ reliability of ~~on~~ DNA recombination techniques is steadily improving ~~progressing~~ in *Escherichia coli* ~~or~~ and coryneform bacteria. ~~In order t~~To improve microorganisms ~~by~~ using DNA recombinant~~tion~~ techniques, ~~even~~ plasmids derived from microorganisms belonging to ~~an~~other species, ~~or~~ genus, or broad host spectrum vectors are often used. However, plasmids ~~proper~~ native to the objective microorganism~~s~~ ~~of improving~~ are generally used. In particular, when the optimum culture temperature for the objective microorganism ~~of the~~ to be improved~~ing~~ is different from that of a microorganism~~s~~ of the same species or genus, it is preferable to use a plasmid ~~proper~~ native to the microorganism.

~~So far~~ To date, ~~obtained as~~ plasmids derived from

coryneform bacteria which have been obtained are pAM330 from *Brevibacterium lactofermentum* ATCC13869 (Japanese Patent Application Laid-open (Kokai) No. 58-67669), pBL1 from *Brevibacterium lactofermentum* ATCC21798 (Santamaria. R. et al., J. Gen. Microbiol., 130, pp.2237-2246, 1984), pHM1519 from *Corynebacterium glutamicum* ATCC13058 (Japanese Patent Application Laid-open (Kokai) No. 58-77895), pCG1 from *Corynebacterium glutamicum* ATCC31808 (Japanese Patent Application Laid-open (Kokai) No. 57-134500) and pGA1 from *Corynebacterium glutamicum* DSM58 (Japanese Patent Application Laid-open (Kokai) No. 9-2603011).

However, no plasmid ~~proper~~ native to *Corynebacterium thermoaminogenes* has been obtained at present.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a plasmid which is useful for improving ~~of the~~ a coryneform bacterium that can grow at an elevated temperature, *Corynebacterium thermoaminogenes*.

The inventors of the present invention found that *Corynebacterium thermoaminogenes* AJ12340 (FERM BP-1539), AJ12308 (FERM BP-1540), AJ12309 (FERM BP-1541) and AJ12310 (FERM BP-1542) each harbored a cryptic plasmid ~~proper~~ native to each strain, and successfully isolated and identified each plasmid. Thus, they accomplished the present invention.

That is, the present invention provides a plasmid isolatable from *Corynebacterium thermoaminogenes*, which

comprises a gene (*rep* gene) coding for a Rep protein which has~~ving~~ the amino acid sequence shown in SEQ ID NO: 2, or an amino acid sequence which has~~ving~~ homology of 90% or more to the foregoing amino acid sequence, and has a size of about 4.4 kb or about 6 kb, or a derivative thereof.

Examples of the aforementioned plasmid include a plasmid isolatable from *Corynebacterium thermoaminogenes* AJ12340 (FERM BP-1539), AJ12308 (FERM BP-1540) or AJ12310 (FERM BP-1542), which has a size of about 4.4 kb and is ~~represented by~~ depicted in the restriction map shown in Fig. 1, and a plasmid isolatable from *Corynebacterium thermoaminogenes* AJ12309 (FERM BP-1541), which has a size of about 6 kb and is ~~represented by~~ depicted in the restriction map shown in Fig. 2.

Specific examples of the aforementioned plasmid include a plasmid which comprises a gene coding for a Rep protein having the amino acid sequence shown in SEQ ID NO: 2, 4 or 6, and a plasmid which comprises a gene coding for a Rep protein having the amino acid sequence shown in SEQ ID NO: 8.

BRIEF EXPLANATION OF THE DRAWINGS

Fig. 1 is a restriction map of the plasmids pYM1, pYM2 and pYM3 of the present invention.

Fig. 2 is a restriction map of the plasmid pYM4 of the present invention.

Fig. 3 shows construction of pYMFK.

Fig. 4 shows construction of pYMK.

Fig. 5 shows construction of pYMC.

Fig. 6 shows construction of pK1.

DETAILED DESCRIPTION OF THE INVENTION

The plasmid of the present invention can be isolated ~~form~~ from *Corynebacterium thermoaminogenes* AJ12340 (FERM BP-1539), AJ12308 (FERM BP-1540), AJ12309 (FERM BP-1541) or AJ12310 (FERM BP-1542) according to a usual method for preparing a plasmid, such as the alkali method (Text for Bioengineering Experiments, Edited by the Society for Bioscience and Bioengineering, Japan, p.105, Baifukan, 1992). ~~As for~~ FERM BP-1539~~, its original deposition, which~~ was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (postal code 305-8566, 1-3 Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) on March 13, 1987 and given an accession number of FERM P-9277~~,~~. This deposit was converted to an ~~was transferred to an~~ international deposit~~ion~~ under the provisions of the Budapest Treaty on October 27, 1987~~and has been deposited at the same depository~~. ~~As for~~ FERM BP-1540, FERM BP-1541 and FERM BP-1542~~, their original depositions, which~~ were deposited at the aforementioned depository on March 10, 1987 and given accession numbers of FERM P-9244, FERM P-9245 and FERM P-9246, ~~were transferred~~ converted to an international deposit~~ions~~ under the provisions of the Budapest Treaty on October 27, 1987~~and have been deposited at the same depository~~.

The inventors of the present invention isolated and identified plasmids ~~each proper~~ native to each of

the aforementioned *Corynebacterium thermoaminogenes* AJ12308 (FERM BP-1540), AJ12310 (FERM BP-1542), AJ12340 (FERM BP-1539) and AJ12309 (FERM BP-1541) ~~from them~~, and designated them as pYM1, pYM2, pYM3 and pYM4, ~~in that order~~ respectively. These plasmids ~~are plasmids that~~ exist as double-stranded circular DNA in a cell of *Corynebacterium thermoaminogenes*. The nucleotide sequence of the *rep* gene contained in pYM1 is shown in SEQ ID NO: 1, the nucleotide sequence of the *rep* gene contained in pYM2 is shown in SEQ ID NO: 3, the nucleotide sequence of the *rep* gene contained in pYM3 is shown in SEQ ID NO: 5, and the nucleotide sequence of the *rep* gene contained in pYM4 is shown in SEQ ID NO: 7. The amino acid sequences that can be encoded by the *rep* genes contained in these plasmids are shown in SEQ ID NOS: 2, 4, 6 and 8. pYM1, pYM2 and pYM3 each have a size of about 4.4 kb. pYM4 has a size of about 6 kb.

The ~~N~~numbers and sizes of fragments that can be obtained when pYM1, pYM2 and pYM3 are digested with typical restriction enzymes are shown in Table 1. The ~~N~~numbers and sizes of fragments that can be obtained when pYM4 is digested with typical restriction enzymes are shown in Table 2. Further, a restriction map of pYM1, pYM2 and pYM3 is shown in Fig. 1, and a restriction map of pYM4 is shown in Fig. 2.

Table 1

Restriction enzyme	Number of digestion site	DNA fragment (kb)
*Bgl*II	0	-
*Bam*HI	2	1.8, 2.6
*Bst*PI	1	4.4
*Eco*RI	1	4.4
*Hinc*II	4	0.3, 0.5, 2.0, 1.6
*Hind*III	0	-
*Kpn*I	0	-
*Nae*I	2	0.1, 4.3
*Nco*I	1	4.4
*Nhe*I	2	1.8, 2.6
*Pma*CI	1	4.4
*Sac*I	0	-
*Sal*I	0	-
*Sac*II	3	0.1, 1.4, 2.9
*Sma*I	3	0.1, 1.8, 2.5
*Sph*I	0	-
*Tth*111I	1	4.4
*Xba*I	0	-

Table 2

Restriction enzyme	Number of digestion site	DNA fragment (kb)
*Bgl*II	1	6.0
*Bam*HI	2	3.8, 2.2
*Bst*PI	2	1.2, 4.8
*Eco*RI	1	6.0
*Hinc*II	4	0.3, 0.4, 1.2, 1.7, 2.4
*Hind*III	0	-
*Kpn*I	0	-
*Nae*I	2	0.1, 5.9
*Nco*I	3	0.2, 2.8, 3.0
*Nhe*I	3	0.1, 2.3, 3.6
*Pma*CI	0	-
*Sac*I	0	-
*Sal*I	0	-
*Sac*II	5	0.1, 0.2, 0.9, 1.8, 3.0
*Sma*I	2	0.1, 5.9
*Sph*I	0	-
*Tth*111I	0	-
*Xba*I	0	-

Determination of the nucleotide sequence of the plasmids of the present invention revealed that pYM1, pYM2, and pYM3 each contained 4368 bp, 4369 bp and 4369 bp, respectively, ~~and they had~~ have substantially the same structure, and ~~showed~~ have homology of 99.9% to one another on the nucleotide sequence level. Further, pYM4 ~~contained~~ contains 5967 bp and ~~it showed~~ has extremely high homology to pYM1, pYM2 and pYM3 ~~for~~ in the ~~region of~~ about 4.4 kb region, ~~except for the region of about 1.6 kb~~, while ~~it showed~~ pYM4 only has homology of about 81% ~~to them~~ when compared as a whole.

The plasmids contain respective *rep* genes which ~~show~~ have high homology to one another. Homology was

compared for the amino acid sequences of the Rep proteins encoded by the *rep* genes (SEQ ID NOS: 2, 4, 6 and 8) and the amino acid sequences of the Rep proteins encoded by *rep* genes of known plasmids derived from coryneform bacteria. Homology of 99% or more was observed among pYM1, pYM2 and pYM3, and homology of 81.91% was observed between pYM2 and pYM4. On the other hand, they showed no homology to the known plasmid pAM330 of a coryneform bacterium, and they showed homology of 80% or less to pGA1 and pCG1. The results are shown in Table 3. Thus, the plasmid of the present invention and the known plasmids of coryneform bacteria are distinguishable based on the homology of the Rep protein.

The homology is calculated according to the method described in Takashi, K. and Gotoh, O., J. Biochem., 92, 1173-1177 (1984).

Table 3

Homology of amino acid sequences of Rep protein encoded by various plasmids

	PYM2	pYM4	pGA1	pCG1
PYM2	-	81.91%	68.01%	70.73%
PYM4	-	-	69.39%	70.23%
PGA1	-	-	-	75.31%
PCG1	-	-	-	-

Since the plasmid of the present invention can sufficiently replicate in cells of coryneform bacteria, including *Corynebacterium thermoaminogenes*, the genetic information of a foreign gene can be expressed in a host microorganism by inserting the foreign gene at any site

~~of~~ in the plasmid, or the derivative thereof, and transforming the host microorganism with the ~~obtained~~ resulting recombinant plasmid.

Examples of coryneform bacteria are listed below.

Corynebacterium acetoacidophilum

Corynebacterium acetoglutamicum

Corynebacterium callunae

Corynebacterium glutamicum

Corynebacterium thermoaminogenes

Corynebacterium lilium (*Corynebacterium glutamicum*)

Corynebacterium melassecola

Brevibacterium divaricatum (*Corynebacterium glutamicum*)

Brevibacterium lactofermentum (*Corynebacterium glutamicum*)

Brevibacterium saccharolyticum

Brevibacterium immariophilum

Brevibacterium roseum

Brevibacterium flavum (*Corynebacterium glutamicum*)

Brevibacterium thiogenitalis

A "derivative" of the plasmid of the present invention means a plasmid composed of a part of the plasmid of the present invention, ~~or a part of the plasmid of the present invention~~ or the plasmid of present invention and another DNA sequence. The "part of a plasmid" means a part containing a region essential for ~~the~~ autonomous replication of the plasmid. The plasmid of the present invention can replicate in a host

microorganism even if a region other than the region essential for the autonomous replication of the plasmid (replication control region), that is, the region other than the region containing the replication origin and genes necessary for the replication, is deleted. In addition, a plasmid ~~including~~ having such a deletion will have ~~has~~ a smaller size. Therefore, a plasmid having such a deletion is preferred for use as a vector. Furthermore, if a marker gene, such as a drug resistance gene, is inserted into the plasmid of the present invention or a part thereof, it becomes easy to detect transformants thanks to the phenotype of the marker gene in the transformants. Examples of such a marker gene that can be used in the host include ~~a~~ chloramphenicol resistance gene, kanamycin resistance gene, streptomycin resistance gene, tetracycline resistence gene, trimethoprim resistance gene, erythromycin resistance gene, and so forth.

Furthermore, if the plasmid of the present invention is made as a shuttle vector, which is autonomously replicable in coryneform bacteria and other bacteria such as *Escherichia coli*, by ligating the plasmid of the present invention or a part thereof with a plasmid autonomously replicable in the other bacteria such as *Escherichia coli* or a part thereof containing a replication control region thereof, manipulations can be performed using *Escherichia coli*, such as preparation of plasmid and preparation of recombinant plasmid containing a target gene ~~can be performed using *Escherichia coli*~~. Examples of ~~the~~ a plasmid

autonomously replicable in *Escherichia coli* include, for example, pUC19, pUC18, pBR322, pHSG299, pHSG298, pHSG399, pHSG398, RSF1010, pMW119, pMW118, pMW219, pMW218, and so forth.

Although pYM1, pYM2, pYM3 and pYM4 ~~themselves~~ are characterized by the restriction maps shown in Figs. 1 and 2, the it is not necessarily required that the plasmid of present invention ~~is not necessarily required to~~ have these restriction maps, and any restriction site may be deleted ~~so~~ as long as such deletion does not affect the autonomous replication ability. Furthermore, the plasmid of the present invention may contain a restriction site that is not contained in pYM1, pYM2, pYM3 and pYM4.

The derivative of the plasmid as described above can be constructed in the same manner as the conventionally known construction of cloning vectors, expression vectors and so forth. In order to construct the derivative, it is preferable to determine the nucleotide sequences of pYM1, pYM2, pYM3 and pYM4. The nucleotide sequences can be determined by known methods, such as the dideoxy method.

In order to insert a foreign gene into the plasmid or the derivative thereof of the present invention, it is convenient to insert it into a restriction site of the plasmid or the derivative thereof. ~~As such a~~ A restriction site~~, one~~ which is present as a single digestion site is preferred. In order to insert a foreign gene, the plasmid and the ~~a~~ source of the foreign gene, such as ~~genome~~ genomic DNA, can be

partially or fully digested with one or more restriction enzymes that provide the same cohesive ends ~~for the both~~, e.g., the same restriction enzyme, and they can be ligated under ~~a~~ suitable conditions. They may also be blunt-end ligated ~~at blunt ends~~.

For the preparation of plasmid DNA, digestion and ligation of DNA, transformation and so forth, ~~those~~ methods well-known to those skilled in the art may be employed. Such methods are described in Sambrook, J., Fritsch, E.F., and Maniatis, T., "Molecular Cloning: A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press (1989), and so forth.

According to the present invention, a novel plasmid derived from *Corynebacterium thermoaminogenes* is provided as described above.

EXAMPLES

Hereinafter, the present invention will be explained in more detail with reference to the following examples.

Example 1

Isolation and characterization of plasmids from *Corynebacterium thermoaminogenes* (FERM BP-1539, FERM BP-1540, FERM BP-1541, FERM BP-1542)

Corynebacterium thermoaminogenes AJ12340 (FERM BP-1539), AJ12308 (FERM BP-1540), AJ12309 (FERM BP-1541) and AJ12310 (FERM BP-1542) were cultured for 12 hours in CM2B liquid medium (Bacto-trypton (Difco): 1%, Bacto-yeast-extract (Difco): 1%, NaCl: 0.5%, biotin: 10 μg/L),

and plasmid DNA fractions were obtained by the alkali method (Text for Bioengineering Experiments, Edited by the Society for Bioscience and Bioengineering, Japan, p.105, Baifukan, 1992). When these fractions were analyzed by agarose gel electrophoresis (Sambrook, J., Fritsch, E.F., and Maniatis, T., "Molecular Cloning: A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press (1989)), DNA bands were detected for all of the ~~cases~~ fractions, and hence it was demonstrated that the aforementioned strains harbored plasmids. The plasmids prepared from FERM BP-1540, FERM BP-1542 and FERM BP-1539 were designated as pYM1, pYM2 and pYM3, respectively. The plasmid prepared from FERM BP-1541 was designated as pYM4. The plasmids pYM1, pYM2 and pYM3 each had a length of about 4.4 kb, and the plasmid pYM4 had a length of about 6.0 kb.

The plasmids pYM1, pYM2, pYM3 and pYM4 were digested with restriction enzymes *Bgl*II, *Bam*HI, *Bst*PI, *Eco*RI, *Hinc*II, *Hin*dIII, *Kpn*I, *Nae*I, *Nco*I, *Nhe*I, *Pma*CI, *Sac*I, *Sac*II, *Sal*I, *Sma*I, *Sph*I, *Tth*111I and *Xba*I (produced by Takara Co.), and the lengths of the produced DNA fragments were measured by agarose gel electrophoresis. The electrophoresis was performed at 100 V/cm and a constant voltage for several hours by using a 0.8% agarose gel. ~~As molecular weight markers,~~ λ phage DNA (Takara Shuzo) digested with a restriction enzyme *Hin*dIII was used as molecular weight markers. The results obtained for pYM1, pYM2 and pYM3 are shown in Table 1. The results obtained for pYM4 are shown in Table 2. The restriction map of pYM1, pYM2 and pYM3 is

shown in Fig. 1, and the restriction map of pYM4 is shown in Fig. 2, which were prepared based on the above results.

The results of nucleotide sequencing of pYM1, pYM2, pYM3 and pYM4 by the dideoxy method are shown in SEQ ID NOS: 1, 3, 5 and 7 ~~in that order~~ respectively.

Example 2

Construction of the shuttle vector pYMFK containing the Km resistance gene derived from *Streptococcus faecalis*

~~As a r~~Regions necessary for efficient replication of pYM2 in coryneform bacteria~~, there are present~~ include an AT-rich region upstream from *rep* and a region which affects~~ing~~ copy number downstream from *rep*, besides the region coding for *rep*.

Therefore, in order to obtain a shuttle vector that can replicate in coryneform bacteria and *E. coli* without impairing the replication ability of pYM2, a region enabling autonomous replication in *E. coli* and a selection marker were inserted into sites in the vicinity of the *Bst*PI site of pYM2.

First, a vector having a drug resistance gene of *S. faecalis* was constructed. The kanamycin resistance gene of *S. faecalis* was amplified by PCR from a known plasmid containing that gene. The nucleotide sequence of the kanamycin resistance gene of ~~the~~ *S. faecalis* has already been elucidated (Trieu-Cuot, P. and Courvalin, P., *Gene*, *23* (3), pp.331-341 (1983)). Based on this sequence, ~~the~~ primers having the nucleotide sequences shown as SEQ ID NOS: 16 and 17 were synthesized, and PCR was performed

~~by~~ using pDG783 (Anne-Marie Guerout-Fleury et al., *Gene*, *167*, pp.335-337 (1995)) as a template to amplify a DNA fragment containing the kanamycin resistance gene and its promoter.

The above DNA fragment was purified by using SUPREC02 produced by Takara Shuzo Co., Ltd., completely digested with restriction enzymes *Hin*dIII and *Hin*cII, and blunt-ended. The blunt-ending was performed by ~~using~~ Blunting Kit produced by Takara Shuzo Co., Ltd. This DNA fragment and an amplification product obtained by PCR ~~utilizing~~ with ~~the~~ primers having the nucleotide sequences shown as SEQ ID NOS: 18 and 19, and pHSG399 (see S. Takeshita *et al.*, *Gene*, *61*, pp.63-74 (1987)) as a template, and purification and blunt-ending ~~of the PCR product~~ were mixed and ligated. The ligation reaction was performed by using DNA Ligation Kit ver.2 produced by Takara Shuzo Co., Ltd. Competent cells of *Escherichia coli* JM109 (produced by Takara Shuzo Co., Ltd.) were transformed with the ligated DNA, and cultured overnight in ~~applied to~~ L medium (10 g/L of Bacto trypton, 5 g/L of Bacto yeast extract, 5 g/L of NaCl, and 15 g/L of agar, pH 7.2) containing 10 μg/ml of IPTG (isopropyl-β-D-thiogalactopyranoside), 40 μg/ml of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and 25 μg/ml of kanamycin~~, and cultured overnight~~. Then, the formed blue colonies were ~~picked up, and~~ subjected to single colony isolation to obtain transformants.

Plasmids were prepared from the transformants ~~by~~ using the alkaline method (Text for Bioengineering Experiments, Edited by the Society for Bioscience and

Bioengineering, Japan, p.105, Baifukan, 1992), and restriction maps were prepared. A plasmid having a restriction map equivalent to that shown at a lower position in Fig. 6 was designated as pK1. This plasmid is stably harbored in *Escherichia coli*, and imparts kanamycin resistance to a host. Moreover, since it contains the *lacZ'* gene, it is suitable for use as a cloning vector.

Then, a region containing the replication origin was amplified by Pyrobest-Taq (Takara Shuzo Co., Ltd.) using pYM2 extracted from *C. thermoaminogenes* AJ12310 (FERM BP-1542) as a template (The entire nucleotide sequence of pYM2 is shown in SEQ ID NO: 9.) and the following primers were prepared based on a sequence in pYM2 near the *Bst*PI site:

S1: 5'-AAC CAG GGG GAG GGC GCG AGG C-3' (SEQ ID NO: 10)

S3: 5'-TCT CGT AGG CTG CAT CCG AGG CGG GG-3' (SEQ ID NO: 11)

The reaction conditions ~~was~~ were 94°C for 5 minutes, ~~then~~ followed by a cycle of 98°C for 20 seconds, and 68°C for 4 minutes, which was repeated for 30 cycles, and 72°C for 4 minutes. After the reaction, the mixture was stored at 4°C.

The ~~obtained~~ resulting amplified fragment was purified ~~by~~ using MicroSpin TM S-400 HR columns produced by Amersham Pharmacia Biotech Co., blunt-ended ~~by~~ using DNA Blunting Kit produced by Takara Shuzo Co., Ltd., and then ligated to pK1, which had been treated with *Hinc*II ~~by~~ using DNA Ligation Kit. ver. 2 produced by Takara Shuzo Co., Ltd. Competent cells of *Escherichia coli*

JM109 (produced by Takara Shuzo) were transformed with the ligated DNA to obtain transformant strains.

Plasmids were prepared from the transformant strains using the alkali method (Text for Bioengineering Experiments, Edited by the Society for Bioscience and Bioengineering, Japan, p.105, Baifukan, 1992) and restriction maps of the plasmids were prepared. ~~One showing a~~A restriction map equivalent to that shown at a lower position in Fig. 3 was designated as pYMFK. pYMFK had a size of about 7.0 kb, and was able to autonomously replicate in *E. coli* and coryneform bacteria and impart Km resistance to a host.

Example 3

Construction of pYMK containing Km resistance gene derived from Tn903

A region containing the replication origin was amplified in the same manner as in Example 2 by using pYM2 extracted from *C. thermoaminogenes* AJ12310 (FERM BP-1542) as a template and the following primers:

S1XbaI: 5'-GCT CTA GAG CAA CCA GGG GGA GGG CGC GAG GC-3' (SEQ ID NO: 12)

S3XbaI: 5'-GCT CTA GAG CTC TCG TAG GCT GCA TCG GAG GCG GGG-3' (SEQ ID NO: 13)

The obtained amplified fragment was purified by using MicroSpin TM S-400 HR columns produced by Amersham Pharmacia Biotech Co., digested with a restriction enzyme *Xba*I produced by Takara Shuzo Co., Ltd., and then ligated to a fragment obtained by fully digesting pHSG299 (Takara Shuzo Co., Ltd.) with *Xba*I by using DNA

Ligation Kit. ver. 2 produced by Takara Shuzo Co., Ltd. Competent cells of *Escherichia coli* JM109 (produced by Takara Shuzo) were transformed with the ligated DNA to obtain transformant strains.

Plasmids were prepared from the transformant strains using the alkali method (Text for Bioengineering Experiments, Edited by the Society for Bioscience and Bioengineering, Japan, p.105, Baifukan, 1992) and restriction maps of the plasmids were prepared. ~~One showing a~~A restriction map equivalent to that shown at a lower position in Fig. 4 was designated as pYMK. pYMK had a size of about 7.0 kb, and was able to autonomously replicate in *E. coli* and coryneform bacteria and impart Km resistance to a host.

Example 4

Construction of shuttle vector pYMC containing Cm resistance gene derived from Tn9

A region containing the replication origin was amplified in the same manner as in Example 2 by using pYM2 extracted from *C. thermoaminogenes* AJ12310 (FERM BP-1542) as a template and the following primers:

S1XbaI: 5'-GCT CTA GAG CAA CCA GGG GGA GGG CGC GAG GC-3' (SEQ ID NO: 14)

S3XbaI: 5'-GCT CTA GAG CTC TCG TAG GCT GCA TCG GAG GCG GGG-3' (SEQ ID NO: 15)

The above DNA was purified by using MicroSpin TM S-400 HR columns produced by Amersham Pharmacia Biotech Co., digested with a restriction enzyme *Xba*I produced by Takara Shuzo Co., Ltd., and then ligated to a fragment

obtained by treating pHSG399 (Takara Shuzo Co., Ltd.) with *Xba*I ~~by~~ using DNA Ligation Kit. ver. 2 produced by Takara Shuzo Co., Ltd. Competent cells of *Escherichia coli* JM109 (produced by Takara Shuzo) were transformed with the ligated DNA to obtain transformant strains.

Plasmids were prepared from the transformant strains using the alkali method (Text for Bioengineering Experiments, Edited by the Society for Bioscience and Bioengineering, Japan, p.105, Baifukan, 1992) and restriction maps of the plasmids were prepared. One showing a restriction map equivalent to that shown at a lower position in Fig. 5 was designated as pYMC. pYMC had a size of about 6.6 kb, and was able to autonomously replicate in *E. coli* and coryneform bacteria and impart Cm resistance to a host.